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EXPERIMENTAL ARTICLES

Amphibacillus fermentum sp. nov. and *Amphibacillus tropicus* sp. nov., New Alkaliphilic, Facultatively Anaerobic, Saccharolytic Bacilli from Lake Magadi

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Abstract—New alkaliphilic, saccharolytic, rod-shaped, gram-positive bacteria resistant to heating and drying and phylogenetically affiliated to the *Bacillus* lineage were isolated under strictly anaerobic conditions from sediments of the alkaline and highly mineralized Lake Magadi. Strain Z-7792 forms endospores; in strain Z-7984, endospore formation was not revealed. The strains are capable of both anaerobic growth (at the expense of fermentation of glucose and certain mono- and disaccharides with the formation of formate, ethanol, and acetate) and aerobic growth. Among polysaccharides, the strains hydrolyze starch, glycogen, and xylan. Yeast extract or methionine are required for growth. The strains are strict alkaliphiles exhibiting obligate requirement for Na^+ and carbonate ions, but not for Cl^- ions. Growth occurs at a total mineralization as high as 3.3–3.6 M Na^+, with an optimum at 1–1.7 M Na^+. Strain Z-7792 is an obligate alkaliphile with a pH growth range of 8.5–11.5 and an optimum of 9.5–9.7. Strain Z-7984 grows in a pH range of 7.0–10.5 with an optimum at 8.0–9.5. Both strains are mesophiles having a growth optimum at 37–38°C. The G+C contents of the DNA of strains Z-7792 and Z-7984 are 39.2 and 41.5 mol %, respectively. These isolates of facultatively anaerobic, strictly alkaliphilic, Na^+-dependent bacilli can be considered representatives of the ecological group adapted to life at drying-up shoals of soda lakes. Because of their independence of NaCl and lack of obligate dependence on sodium carbonates, the isolates are to be assigned to athalassophilic organisms. According to their physiological and phylogenetic characteristics, they taxonomically belong to group 1 of the species of bacilli with a low G+C content and occupy a position intermediate between the genera *Amphibacillus* and *Gracilibacillus*. The isolates are described as new species of *Amphibacillus: A. fermentum* (type strain, Z-7984^T) and *A. tropicus* (type strain, Z-7792^T).

Key words: soda lakes, alkaliphiles, facultative anaerobes, saccharolytic bacteria, *Bacillus*, xylanolytics.

The genus *Bacillus* comprises both aerobic and facultatively anaerobic bacteria. The species of this genus have been isolated from various habitats, among them extreme habitats, including soda environments with their high alkalinity.

Alkaliphilic representatives of the genus *Bacillus* were among the first organisms investigated in soda environments. This fact was due to the increased interest in these bacteria as possible producers of alkaline hydrolytic enzymes for the industry [1] and as model organisms to study the bioenergetics of alkaliphiles [2] and osmoregulation in haloalkaliphiles [3].

As spore-forming organisms, bacilli are indispensable components of the microbiota of soda saline soils, since this microbiota is adapted to sharp changes in the mineralization of soil solution and to drying. On the contrary, in bottom sediments of soda lakes, the conditions are humid and anaerobic, although seasonal drying and transition to a state of "dry lakes" are characteristic of many soda lakes. So far, all of the bacilli isolated from soda lakes are strictly aerobic [3–6] and are considered to be the major component of the microflora of the aerobic zone. It remained unclear whether facultatively anaerobic bacilli can be involved in the trophic system of the anaerobic alkaliphilic community, for which only obligate anaerobes (although varying in their metabolism) have been described [7].

While studying carbohydrate degradation by alkaliphiles under strictly anaerobic conditions, we found, in addition to spirochetes [8], spore-forming saccharolytics affiliated to the phylogenetic lineage of the low G+C gram-positive bacteria and belonging to its different taxonomic subgroups [9]: gram-negative haloanaerobes (type species, *Halonatronum saccharophilum* [10]) and gram-positive clostridia and bacilli. The bacillar strains Z-7792 and Z-7984 proved to be able not only to ferment carbohydrates but also to grow aerobically; thus, they are facultative anaerobes.

According to data from preliminary sequencing of 16S rRNA (400 nucleotides), strains Z-7984 and

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Z-7792 were assigned to group 1 [11] of the spectrum of *Bacillus* species, where they occupied an independent position; it was recommended that these strains should be described as new taxa within this group [9]. In addition to presenting taxonomic interest, facultatively anaerobic alkaliphilic bacilli are also of interest as members of the anaerobic alkaliphilic community and as representatives of the microbiota of drying-up soda lakes. In the present work, we describe these bacilli, isolated from the continental highly mineralized soda Lake Magadi, as a new species of the genus *Amphibacillus*, *A. fermentum* sp. nov. and *A. tropicus* sp. nov.

MATERIALS AND METHODS

Isolation source. The isolation source was bottom sediment of a coastal lagoon of Lake Magadi (Kenya). Strain Z-7984, like *Halonatronum saccharophilum* [10], was isolated from sample no. 6 taken by G. Zavarzin in September 1998 (dry period, when the water in the lake was saturated with soda, had a pH of 10.2, a mineralization of 260 g/l, and a temperature of 39°C). Strain Z-7792 was isolated from sample no. 3 taken from the same lagoon in May 1992 (rainy period, when the water had a mineralization of 160 g/l and the pH and temperature were the same as in September 1998).

Cultivation conditions. Enrichment cultures were obtained under strictly anaerobic conditions on modified mineral medium 1 [12] having pH 10 and a total mineralization of 160 g/l and containing 2.65 M Na^+ (0.9 M NaCl, 0.6 M Na_2CO_3, 0.55 M $NaHCO_3$) and 5 g/l of glucose as the substrate.

The optimized medium for the anaerobic cultivation of both strains had the following composition (g/l): KH_2PO_4, 0.2; $MgCl_2$, 0.1; NH_4Cl, 0.5; KCl, 0.2; Na_2CO_3, 63.6; $NaHCO_3$, 50.4; trace element solution [10], 1 ml/l; resazurin, 0.001; $Na_2S \cdot 9H_2O$, 0.7; yeast extract, 0.2; sucrose, 5.0 (pH 9.5). Nitrogen was the gas phase. When cultivation was performed under aerobic conditions, $Na_2S \cdot 9H_2O$ and resazurin were omitted.

To establish the spectrum of catabolic substrates utilized, they were added at concentrations of 3 g/l for cultivation against the background of 0.2 g/l of yeast extract. Sterile aqueous solutions of sugars were added to the alkaline medium immediately before inoculation.

Determination of physiological characteristics. Electron acceptors were introduced into a sterile medium to the following concentrations (mM): $Na_2S_2O_4$, 1; Na_2SO_3, 2 or 10; $NaNO_3$, 10; Na_2SO_4, 10; $Na_2S_2O_3 \cdot 5H_2O$, 10 or 20; S^0, 2%. Utilization of sulfur compounds as electron acceptors was determined from hydrogen sulfide formation; as the reducing agent, sodium thioglycollate was used. Capacity for nitrogen fixation was determined in a nitrogen-free medium. In the experiments on the pH dependence of growth, 10% HCl or 10% NaOH was used to adjust pH of medium 1 in which the carbonate concentration was decreased tenfold, the only carbonate species was sodium bicarbonate, and optimal sodium concentration was maintained with sodium chloride. The requirement for sodium carbonates was studied in a medium in which they were replaced with an equimolar amount of NaCl and where pH 9.0 was maintained with a 50 mM serine buffer. The requirement for chloride ion was studied in a medium in which NaCl was replaced with an equimolar amount of sodium carbonate and sodium bicarbonate and all other chlorides were replaced with sulfates. The dependence of growth on temperature was studied in a temperature range from 6 to 65°C at optimal pH and mineralization. Cell resistance to drying was studied by two methods simulating the conditions occurring in soda lakes during their evaporation in the dry period. In the first variant, 0.5 ml of a culture grown in medium 1 was applied onto a strip of filter paper, dried under aerobic conditions at 60°C, and introduced into an optimal growth medium. In the second variant, cells were grown at a maximum mineralization, and then 0.5 to 1 ml of the culture was gradually evaporated at 60°C for two weeks. Then, salt crystals with embedded cells were flooded with fresh medium optimal for growth. In both cases, resumption of growth was monitored. The requirement for individual amino acids was determined by successively omitting one of the 20 major amino acids (the concentration of each amino acid was 20 mg/l).

Analytical methods. Growth was determined by measuring optical density of the culture in Hungate tubes on a Specol-10 (Jena) spectrophotometer at 600 nm. Glucose was analyzed in a reaction with phenol [13]. Hydrogen and nitrogen were quantitatively determined on an LKhM-80 gas chromatograph equipped with a katharometer. Volatile fatty acids were analyzed on a model 3700 gas chromatograph equipped with a flame ionization detector. Formate was determined colorimetrically [14]. Dissolved hydrogen sulfide was determined colorimetrically from the formation of methylene blue [15]. The presence of catalase was judged from the foaming of a drop of 3% hydrogen peroxide on the addition of biomass washed three times in 10% NaCl to remove carbonates.

Morphological studies. Light-microscopic examination of cell morphology was performed using a Zetopan (Austria) phase-contrast microscope. Agarose slides were used when taking photographs [16]. Ultrathin sections were obtained as described earlier [10]. The sections and whole cells stained with 1% phosphotungstic acid to reveal flagella were examined under a JEM-100C (Japan) electron microscope.

Determination of nucleotide sequences of the 16S rRNA genes. Amplification and sequencing of 16S rRNA gene fragments were performed as described earlier [9].

Analysis of the nucleotide sequences of the 16S rRNA genes. Nucleotide sequences of the 16S rRNA genes of strains Z-7984 and Z-7792 were manually

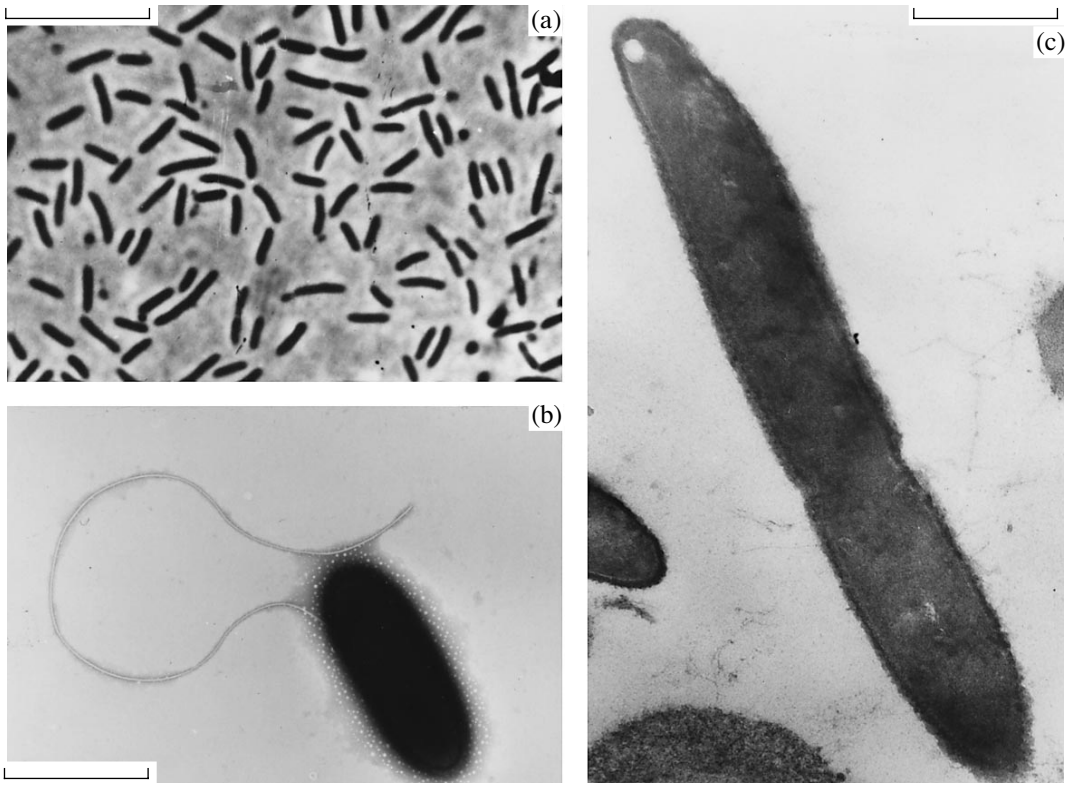

Fig. 1. Morphology of strain Z-7984 cells: (a) vegetative midlog-phase cells under phase contrast microscope; bar, 10 μm; (b) a cell with a subterminal flagellum under an electron microscope; bar, 1 μm; (c) a longitudinal ultrathin section of a vegetative cell; bar, 0.5 μm.

aligned (using a BIOEDIT sequence editor) with 16S rRNA gene sequences of group 1 bacilli. Unrooted phylogenetic trees were constructed using various algorithms implemented in the TREECON and PHYLIP software packages [10].

The 16S rDNA sequences of strains Z-7984 and Z-7792 were submitted to GenBank under accession numbers AF418603 and AF418602, respectively.

RESULTS

Isolation. Both strains were isolated under strictly anaerobic conditions at 37°C on highly mineralized alkaline (pH 10) medium 1 with glucose (for strain Z-7792) or sucrose (for strain Z-7984) as the substrate in the presence of yeast extract. In the enrichment culture of stain Z-7792, spores were revealed. Pasteurization at 80°C for 20 min allowed a part of the satellite microflora to be eliminated, and strain Z-7792 became a dominant form in the culture. In the pure culture of strain Z-7984, spore formation was not revealed. Pure cultures were obtained by inoculating the liquid medium with serially diluted inoculum and subsequent plating of grown cultures on an agarized medium.

The colonies of strain Z-7984 were yellowish, circular, with a transparent center and raised denser margins. The colony diameter was 0.5 to 1.5 mm after 3 days of cultivation. The colonies of strain Z-7792 were white, tinged with pink, circular, slightly convex, with a smooth surface, transparent center, and denser, and sometimes uneven, margins. The colony diameter was 1 mm after 3 days of cultivation. Microscopically homogeneous cultures of strains Z-7984 and Z-7792 grown from single colonies were taken for further study.

Morphology and ultrastructure. Cells of both strains were thin motile rods; however, they were morphologically distinguishable.

Cells of strain Z-7984 were short rods with somewhat sharpened ends, occurring usually singly or in pairs or sometimes in short chains of up to 6 cells. In the exponential growth phase, which lasted 13 h on average, the cells of strain Z-7984 measured 0.5–0.75 μm in diameter and 1.5–4 μm in length (Fig. 1). Spore formation was not observed in this strain, but it exhibited heat resistance: growth resumed after heating cells at 80°C for 50 min, although after a prolonged lag phase (up to 5 days).

Cells of strain Z-7792 taken from the exponential phase (which lasted 10 h on average) were thinner and longer, measuring 0.4–0.5 μm in diameter and 2–6 μm in length (Fig. 2). At the end of the exponential phase, spores could be observed in some cells. The spores were terminal, oval, about 1 μm in diameter (Fig. 2a); they could withstand heating at 90°C for 10 min (when

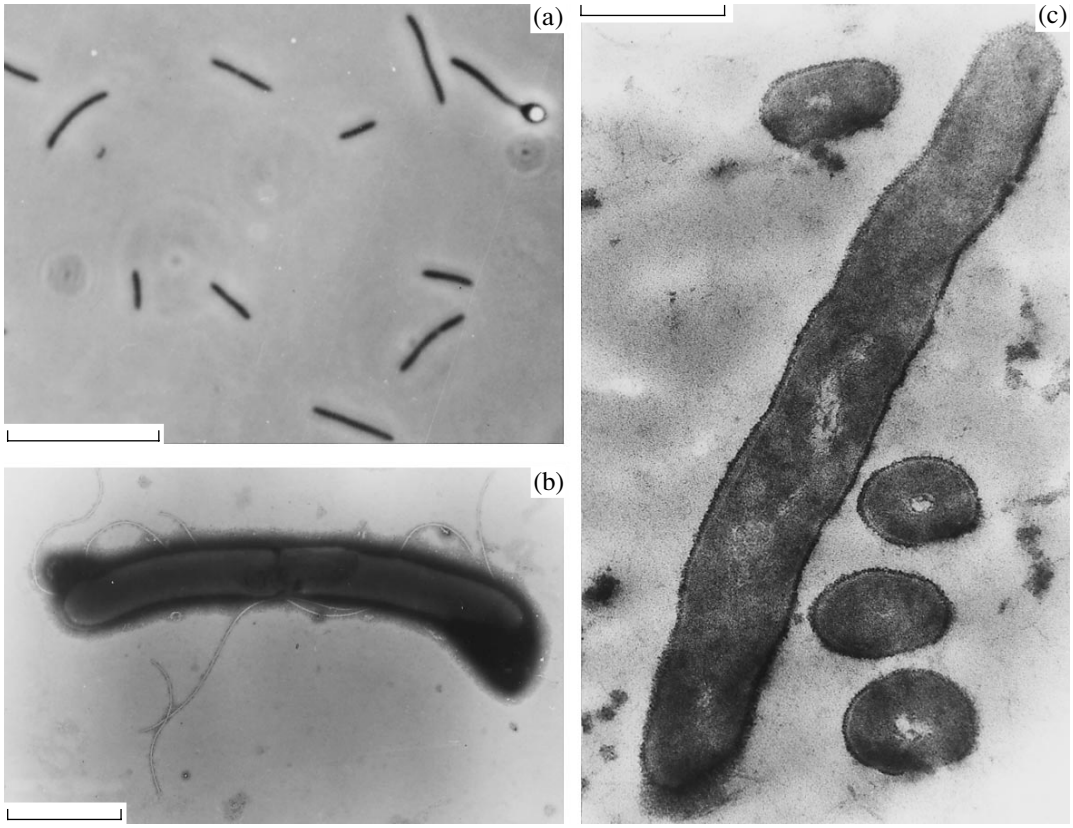

Fig. 2. Morphology of strain Z-7792 cells: (a) vegetative cells and a cell with a mature spore (midlog phase) under phase contrast microscope; bar, 10 μm; (b) a cell with peritrichous flagella under an electron microscope; bar, 1 μm; (c) a longitudinal ultrathin section of a cell and several transversely sectioned cells; bar, 0.5 μm.

heated spores were used as the inoculum, growth started on the fourth day). In the stationary phase, a part of cells lysed with the formation of spheroplasts.

Cells of both strains multiplied by binary fission, which was sometimes not quite symmetrical. Cells of both strains were motile; the flagellation types were, however, different. In strain Z-7792, the flagellation was peritrichous (Fig. 2b). Cells of strain Z-7984 had one subterminal flagellum (Fig. 1b). By the type of their cell wall structure, both strains belonged to gram-positive bacteria (Figs. 1c, 2c).

Growth characteristics. Strain Z-7984 grew at a mineralization of the medium of 0.17 to 3.3 M Na^+ with an abrupt cessation of growth at 3.4 M Na^+. The curve of the dependence of the specific growth rate on mineralization has a broad plateau in the region of 0.67–3.1 M with a weakly pronounced maximum at 1.87 M (Fig. 3a). The bacterium grew at alkaline pH values (7.0–10.5) with a broad optimum at pH 8.0–9.5. At pH 6.5 or 10.9 no growth occurred (Fig. 3b). At optimal values of pH and mineralization, the temperature optimum was recorded at 36–38°C; the growth range was 18–56°C (Fig. 3c). No growth occurred at 6 or 60°C.

Strain Z-7792 grew in a somewhat broader range of mineralization values, from 0.17 to 3.6 M; however, the growth of this strain exhibited a sharp optimum at 1–1.87 M Na^+ (Fig. 4a). Growth occurred at pH 8.5–11.5 with a pronounced optimum at 9.5–9.7. No growth occurred at pH 8.0 or 12.0 (Fig. 4b). The temperature range for growth was 18–56°C; the optimum was 38°C (Fig. 4c).

Neither of the strains required chloride ions: growth occurred after the replacement of NaCl with an equimolar amount of Na_2CO_3 + $NaHCO_3$. Both strains showed an obligate requirement for Na_2CO_3 + $NaHCO_3$: no growth occurred after the replacement of carbonates with an equimolar amount of NaCl (the pH in this experiment was maintained with 50 mM serine buffer (pK_a = 9.2)). Na^+ was also obligately required by both strains: the replacement of sodium ions with potassium ions resulted in the lack of growth.

Strains Z-7984 and Z-7792 are facultative anaerobes. Both are catalase-positive. During anaerobic growth, both thioglycollate and sodium sulfide were appropriate reducing agents. Cysteine could be used as the reducing agent, and it was also utilized as the source of sulfur and nitrogen. The strains are incapable of nitrogen fixation or of dissimilatory reduction of nitrate or sulfur compounds (SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$); however, they reduced S^0 with the formation of H_2S (28.3 mM

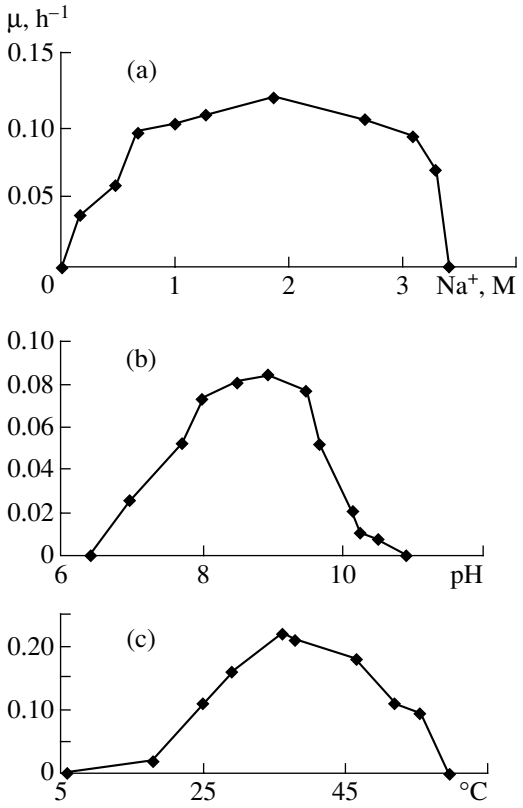

Fig. 3. Dependence of the specific growth rate of strain Z-7984 on (a) mineralization of the medium, (b) pH, and (c) temperature.

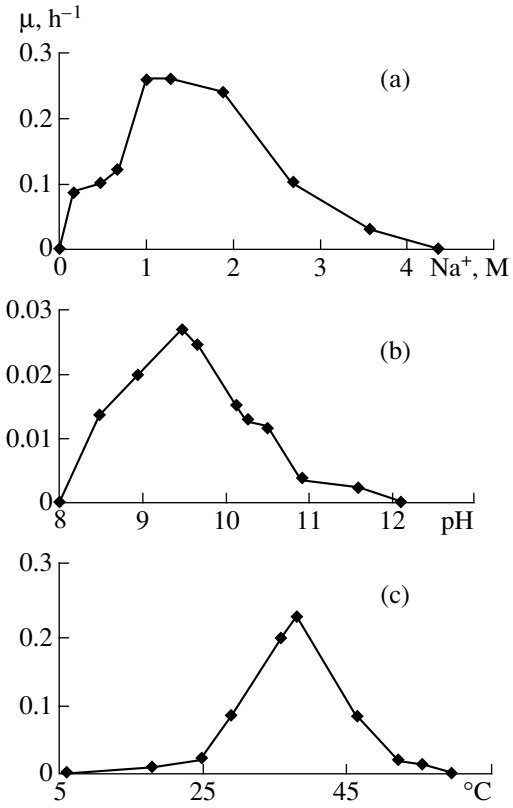

Fig. 4. Dependence of the specific growth rate of strain Z-7792 on (a) mineralization of the medium, (b) pH, and (c) temperature.

was formed by strain Z-7984, and 8.3 M by strain Z-7792). The tolerance to sulfide was different: 28 mM hydrogen sulfide added to the medium completely inhibited the growth of strain Z-7792 but did not inhibit the growth of strain Z-7984 (although the lag phase became much longer). Other electron acceptors tested, as well as sodium dithionite, neither inhibited nor stimulated growth. The revealed tolerance to sulfide allows these facultatively anaerobic organisms to develop in the zone of active sulfidogenesis.

The energy sources utilized by strain Z-7984 during anaerobic growth were D-xylose, D-glucose, D-mannose, D-fructose, L-sorbitol, sucrose, D-maltose, trehalose, and D-cellobiose. Utilization of peptone, yeast extract, and Tween 80 was weak. Starch, glycogen, and xylan were hydrolyzed. During aerobic growth, strain Z-7984 could additionally utilize D-ribose, D-galactose, L-arabinose, D-lactose, and *N*-acetylglucosamine, but not Tween 80 (Table 1).

Strain Z-7792 could ferment anaerobically D-glucose, sucrose, D-maltose, trehalose, D-cellobiose, melibiose, peptone, yeast extract, and, to some extent, Tween 80. Starch, glycogen, and xylan were hydrolyzed. During aerobic growth, strain Z-7792 could additionally utilize D-fructose, D-xylose and D-lactose but not yeast extract, peptone, or Tween 80 (Table 1).

The strains did not grow on other hexoses, pentoses, disaccharides, or sugar alcohols or on microcrystalline cellulose, mono- and dicarboxylic organic acids, monohydroxylic alcohols, Casamino acids, or casein. The strains did not liquify gelatine or agar (see note to Table 1). Both strains required yeast extract, which could be replaced by Casamino acids. The growth yield of the strains was directly related to the concentration of these compounds within the range of 200–1000 mg/l. The only amino acid obligately required was methionine. However, the substitution of methionine (0.2 g/l) or an amino acid mixture for yeast extract prolonged the lag phase and notably decreased the growth rate. Most probably, yeast extract serves not only as an amino acid source but also as a source of other growth factors whose nature remains unknown.

The products of glucose fermentation by both strains were formate, acetate, ethanol, and, judging from carbon and hydrogen balance considerations, carbon dioxide. From 18.0 mM glucose, strain Z-7984 formed 14.6 mM acetate, 7.6 mM ethanol, and 55.4 mM formate; the carbon balance was 92.3%, and the hydrogen balance was 99.4%. Strain Z-7792 formed, from 12.3 mM glucose, 5.3 mM acetate, 7.3 mM ethanol, and 50.4 mM formate; the carbon balance was 95.3%, and the hydrogen balance was 112%. Under optimal

Table 1. Substrate utilization by strains Z-7984 and Z-7792

Substrate	Maximum optical density, λ = 600 nm			
	Z-7984		Z-7792	
	anaerobic growth	aerobic growth	anaerobic growth	aerobic growth
Pentoses				
D-ribose	0	0.110	0	0
D-xylose	0.100	0.110	0	0.060
L-arabinose	0	0.100	0	0
Hexoses				
D-glucose	0.120	0.100	0.080	0.090
D-mannose	0.130	0.100	0	0
D-fructose	0.200	0.110	0	0.090
D-galactose	0	0.060	0	0
Sugar alcohols				
L-sorbitol	0.210	0.060	0	0
Disaccharides				
D-lactose	0	0.080	0	0.080
sucrose	0.290	0.090	0.070	0.090
melibiose	0	0	0.050	0.050
D-maltose	0.240	0.140	0.075	0.160
trehalose	0.250	0.070	0.085	0.160
D-cellobiose	0.270	0.110	0.100	0.160
Polysaccharides				
starch	0.220	0.150	0.120	0.250
glycogen	0.330	0.085	0.150	0.075
xylan	0.090	0.050	0.090	0.030
Esters of fatty acids and sorbitol				
Tween 80	0.025	0	0.025	0
Nitrogen compounds				
N-acetylglucosamine	0	0.090	0	0
peptone	0.045	0	0.085	0
yeast extract	0.035	0	0.065	0

Note: In addition to the substrates listed in the table, the following substrates were tested (with negative results for both strains): D-fucose, sorbose, glycerol, mannitol, dulcitol, L-inositol, erythritol, methanol, ethanol, formate, acetate, propionate, butyrate, glycolate, lactate, pyruvate, malonate, succinate, choline chloride, betaine, trimethylamine, microcrystalline cellulose, pectin, gum arabic and Casamino acids. Gelatine and agar were not hydrolyzed.

growth conditions, the generation times were 3.0 and 3.15 h for strains Z-7792 and Z-7984, respectively.

Both strains were resistant to drying. However, cells of strain Z-7984 were only resistant to drying on filter paper, whereas experiments with strain Z-7792 showed that both its cells dried on filter paper and its cells incorporated into salt crystals that had formed during evaporation of highly mineralized medium could resume growth.

DNA analysis. The G+C contents of the DNA of strains Z-7984 and Z-7792, determined from the melting temperatures, were 41.5 and 39.2 mol %, respectively. The DNA–DNA homology between the strains was 35% [9].

Phylogenetic analysis. Almost complete sequences of the 16S rRNA genes of strains Z-7984 and Z-7792 were determined: 1492 nucleotides (between *E. coli* positions 41 and 1553) for strain Z-7984 and 1461 nucleotides (between *E. coli* positions 48 and 1509) for strain Z-7792. Comparative analysis of these sequences with the GenBank BLAST program confirmed our initial conclusion about the affiliation of the bacteria stud-

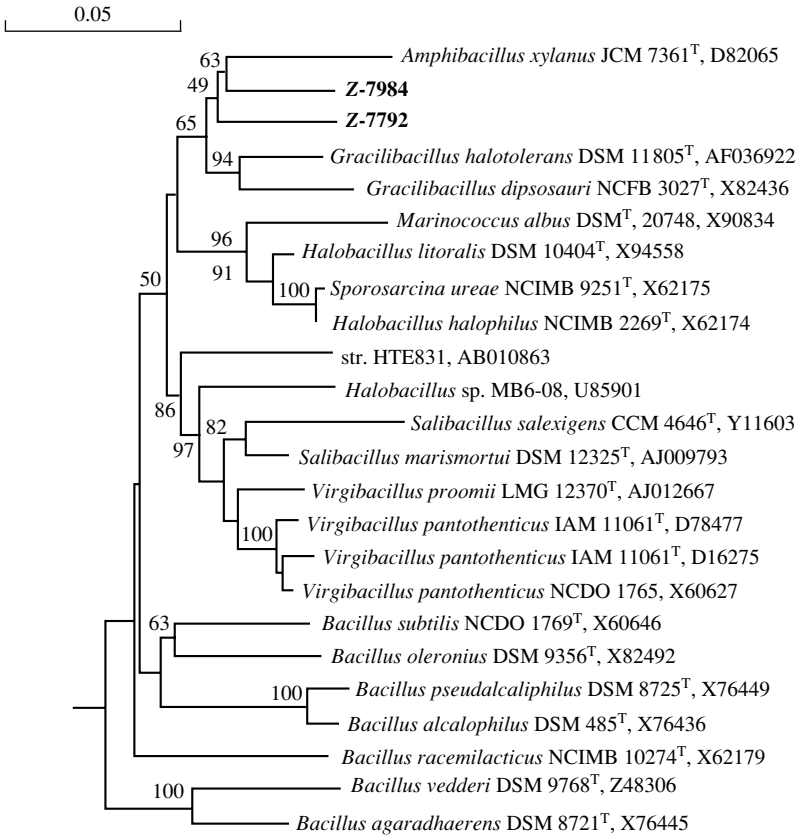

Fig. 5. Unrooted phylogenetic tree of bacilli constructed based on the comparison of nucleotide sequences of the 16S rRNA genes. Bar corresponds to 5 nucleotide substitutions per 100 nucleotides. Figures at the branching points show the statistical significance of the branching order as determined by bootstrap analysis (values higher than 45 were considered significant).

ied with group 1 of the species of bacilli [9]. In the phylogenetic tree (Fig. 5) including representatives of group 1 of the species of bacilli, as well as alkaliphilic and haloalkaliphilic bacilli, strains Z-7984 and Z-7792 belong to the phylogenetic subgroup of *Virgibacillus pantothenicus.* In this subgroup, strain Z-7984 clusters with the alkaliphilic species *Amphibacillus xylanus*, although the homology of sequences is not very high (94.6%) and the value from the bootstrap analysis is as low as 63%. Strain Z-7792 occupies an intermediate position between the species *A. xylanus* and *Gracilibacillus halotolerans*; however, this position is supported by a bootstrap value as low as 49%. The strains studied exhibit the highest 16S rDNA homology with the extremely halotolerant species of the genus *Gracilibacillus*, *Gracilibacillus halotolerans* (95.2 and 95.6% for strains Z-7984 and Z-7792, respectively). The 16S rDNA homology between strains Z-7984 and Z-7792 is 95.4%. With other members of group 1 of the species of bacilli, the new isolates have homology values from 91.7 to 94.6%. With obligately alkaliphilic representatives of group 6 (*Bacillus vedderi, B. agaradhaerens, B. alcalophilus,* and *B. pseudalcaliphilus*), strains Z-7984 and Z-7792 have homology values of 90.5% to 93.4%.

DISCUSSION

The chemoorganotrophic, saccharolytic, facultatively anaerobic bacilli that we isolated proved to be able to compete with obligately anaerobic bacteria in enrichment cultures grown anaerobically on easily degradable carbohydrates. Under natural conditions, the competitive ability of bacilli should be even greater due to their resistance to drying (strain Z-7792 could even germinate from the crystals of dried highly mineralized medium). Such resistance is important for the inhabitants of lake shoals undergoing periodical seasonal drying.

A high osmoadaptation capacity is another prerequisite for microorganism persistence in soda saline soils and shoals of soda lakes. Indeed, strain Z-7984 exhibited a remarkably high growth rate in a wide mineralization range. Independence of chloride ions and obligate requirement for sodium ions indicate an athalassic origin of these bacilli, as distinct from the marine origin of the strictly anaerobic haloalkaliphilic isolates from the same habitat, *Halonatronum* [10] and *Spirochaeta africana* [8].

Thus, the alkaliphilic bacilli we isolated are organisms adapted to the existence in periodically drying

Table 2. Comparison of the type species of the *Virgibacillus pantothenicus* subgroup of group 1 of the species of bacilli

Characteristics	*Virgibacillus pantothenicus*^{T}	*Salibacillus salexigens*^{T}	*Halobacillus halophilus*^{T}	*Gracilibacillus halotolerans*^{T}	*Gracilibacillus dipsosauri*	*Amphibacillus xylanus*^{T}	Z-7984	Z-7792
Morphology	Rods	Rods	Spheres or ovals	Rods	Rods	Rods	Rods	Rods
Motility	+	+	+	+	+	Lacking in type strain	+	+
Colony pigmentation	Grayish white	Nonpigmented	Orange	Creamy white	White	White	Yellowish	Pinkish white
Spore shape	Ellipsoidal or spherical	Oval	Ellipsoidal	Ellipsoidal	Spherical	Oval	Spores not revealed	Ellipsoidal
Spore location	Terminal or subterminal	Central or subterminal	Central	Terminal	Terminal	Terminal		Terminal
Anaerobic growth	+	–	–	–	+	+	+	+
$S^0 \longrightarrow H_2S$	ND	–	–	–	ND	–	+	+
Catalase	+	+	+	+	+	–	+	+
NaCl, % (optimum)	0–10 (4)	10–20 (10)	2–15 (3–5)	0–20 (0)	0–15 (ND)	ND	0.98–19.7 (10.8)	0.98–20.9 (5.4–10.8)
pH (optimum)	ND	6.0–11.0 (7.5)	7.0–9.0 (7.8)	5.0–10.0 (7.5)	6.5–10.0 (7.5)	8.0–10.0 (ND)	7.0–10.5 (8.5–9.0)	8.5–11.5 (9.5–9.7)
temperature range (optimum)	28–45 (37)	15–45 (37)	15–37 (30)	6–50 (47)	28–50 (45)	25–45 (ND)	18–56 (36–38)	18–56 (38)
Nitrate reduction	Lacking in type strain	–	–	+	+	–	–	–
Substrates utilized								
D-arabinose	+	ND	–	+	ND	+	+	–
D-ribose	+	+	ND	ND	ND	+	+*	–
D-xylose	–	+	–	+	ND	+	+	+
D-glucose	+	+	–	+	+	+	+	+
D-fructose	+	+	–	+	ND	+	+	+

Table 2. (Contd.)

Characteristics	*Virgibacillus pantothenicus*^{T}	*Salibasillus salexigens*^{T}	*Halobacillus halophilus*^{T}	*Gracilibacillus halotolerans*^{T}	*Gracilibacillus dipsosauri*	*Amphibacillus xylanus*^{T}	Z-7984	Z-7792
Substrates utilized								
D-mannose	+	+	ND	+	ND	+ (type strain)	+	–
D-galactose	+	–	–	+	ND	ND	+*	–
D-mannitol	–	+	–	+	+	ND	–	–
glycerol	+	+	ND	+	+	ND	–	–
L-sorbitol	ND	ND	ND	ND	ND	ND	+	–
D-fucose	+	ND	ND	–	+	ND	–	–
D-lactose	+	–	ND	+	ND	ND	+*	+*
sucrose	+	–	–	ND	+	+	+	+
melibiose	–	–	ND	+	ND	+	–	+
D-maltose	+	+	–	+	ND	+	+	+
trehalose	+	–	–	+	ND	+	+	+
D-cellobiose	+	+		+	ND		+	+
glycogen	–	ND	ND	+	+	ND	+	+
xylan	ND	ND	ND	ND	ND	+	+	+
starch	+	–	+	+	+	+	+	+
Tween 80	–	–	–	+	ND	ND	+**	+**
N-acetylglucosamine	+	+	ND	+	ND	ND	+*	–
G+C, mol %	36.9	39.5	40.8	38.0	39.4	36.0	41.5	39.2
Isolation source	Soil, southern England	Salterns and hypersaline soils, Spain	North Sea coast, Germany	Great Salt Lake, Utah, USA	Nasal salt glands of *Dipsosaurus dorsalis*	Rice straw compost	Lake Magadi	

Note: "ND" means "no data."
* The substrate is used only under aerobic conditions.
** The substrate is used only under anaerobic conditions.

zones of soda lakes. In these habitats, they represent terrestrial microflora.

Unlike clostridia, bacilli belong to the group of aerobic organisms. It is known that phylogenetic systematics rarely allows conclusions to be made as to physiological properties of the organisms. However, in our case, the phylogenetic position of our anaerobic isolates Z-7792 and Z-7984 prompted us to investigate their ability to grow aerobically. Interestingly, these facultatively anaerobic bacilli could not reduce exogenous electron acceptors and, under anaerobic conditions, switched to fermentation with the formation of formate as the main product. Formate can be easily used in the anaerobic community [7] in a process analogous to the interspecies hydrogen transfer.

The taxonomy of the species of bacilli is currently reconsidered with taking into account the nucleotide sequences of their 16S rRNA genes [11, 17]. Phylogenetic heterogeneity of the genus *Bacillus* was established, and this resulted into its subdivision into groups and to description of new genera and species within these groups. As a result of the investigation of industrially important alkaliphilic and alkalitolerant aerobic bacilli, group 6 was distinguished, which includes *Bacillus alcalophilus* and some bacilli isolated from soils [17]; afterwards, nine new species were suggested [18].

In soda lakes, which are inhabited by diverse microflora [5–7], bacilli were considered to be members of the aerobic community [5, 6], since the few strains isolated were strictly aerobic. The only species described was the obligately alkaliphilic *Bacillus haloalkaliphilus* isolated from Wadi Natrun (Egypt) [3, 4]. The isolates from Kenyan soda lakes have not yet been formally described; however, their phylogenetic investigation showed that some of them are close to group 6 and that the Na^+-dependent strains belong to group 7 [5, 19], which includes *Bacillus agaradhaerens* and *Bacillus clarkii* [18]. It is believed that the habitats of group 6 bacilli are confined to near-shore soil, whereas strains of group 7 inhabit lake water and sediments, where the conditions are less variable [5]. It is noteworthy that the strictly alkaliphilic Lake Magadi isolates Z-7792 and Z-7984, which are obligately dependent on sodium and carbonate ions but are independent of chloride ion, proved to belong phylogenetically to group 1 [11] (*Virgibacillus pantothenicus* subgroup). This group includes alkalitolerant, halophilic or halotolerant, aerobic bacilli (Table 2); the only exception is *Amphibacillus xylanus* [20], an obligately alkaliphilic facultatively anaerobic organism isolated from rice straw compost. In group 1, our isolates occupy a position intermediate between *Amphibacillus* and the recently established genus *Gracilibacillus* [24], which includes extremely halotolerant species. Both the phenotypic properties of the new isolates (Table 2) and their phylogenetic position erode the hiatus between these two new genera and suggest their merging in a large genus. By their physiological characteristics (capacity for anaerobic growth, pH optimum higher than 8.5, ability to hydrolyze xylan, starch, and glycogen), the new isolates are closer to *Amphibacillus xylanus* than to *Gracilibacillus halotolerans*, a species which is extremely halotolerant, aerobic, and alkalotolerant. At the same time, the new isolates differ from the species of both of the above two genera by obligate requirement for sodium carbonates and by populating another habitat.

Seeking to avoid excessive splitting of genera, we suggest that the alkaliphilic facultatively anaerobic bacilli we isolated be assigned to the genus *Amphibacillus*. Evidently, there are sufficient phylogenetic (5% dissimilarity level, Table 2, Fig. 5), genetic (35% homology), and phenotypic (Table 1) grounds to assign the isolates to new species, whose descriptions are presented below.

Description of *Amphibacillus fermentum* sp. nov. (fer.men.tum, from L. n. *ferment*, fermentation; adj. *fermentum*, fermenting organism).

Cells are rods measuring 0.5–0.75 by 1.5–4 μm and occurring singly, in pairs, or, sometimes, in short chains. Motile by means of one subterminal flagellum. The cell wall is of the gram-positive type. Spores were not revealed, but the cells are heat-resistant.

Strictly alkaliphilic. Grows within the pH range of 7.0–10.5 with an optimum at pH 8.0–9.5. Obligately dependent on CO_3^{2-} ion. Grows at a total mineralization of 0.17–3.3 M Na^+ with an optimum of 1.87 M Na+ (in the form of sodium carbonates). Cl^- ion is not required.

Mesophilic. The range of growth temperatures is 18–56°C, the optimum temperature is 36–38°C. Under optimal growth conditions, the generation time is 3.15 h.

Facultatively anaerobic. Catalase-positive. During anaerobic growth, ferments glucose, xylose, mannose, fructose, sucrose, maltose, trehalose, cellobiose, and, at a low rate, peptone, yeast extract, and Tween 80. The products of glucose fermentation are formate, acetate, and ethanol. Starch, glycogen, and xylan are hydrolyzed. Additional substrates utilized during aerobic growth are ribose, arabinose, galactose, lactose, and *N*-acetylglucosamine.

Chemoorganotrophic. Exhibits an anabolic requirement for yeast extract (the obligate requirement is methionine). Uses sulfur as an electron acceptor. Tolerant to sulfide. Sulfur reduction is not coupled to energy generation.

The G+C content of DNA is 41.5 mol %.

Isolated from the bottom sediment of a coastal lagoon of Lake Magadi (Kenya).

The type strain is Z-7984^T (=DSM 13869; Uniqem 210).

Description of *Amphibacillus tropicus* sp. nov. (tro.pi.cus; L. adj. *tropicus*, tropical; an organism isolated from a tropical lake).

Cells are thin rods measuring 0.4–0.5 by 2–6 μm and occurring singly or in pairs. Motile by means of peritrichous flagella. The cell wall is of the gram-positive type. Endospores are formed, which are oval, terminal, heat-resistant.

Strictly alkaliphilic. Grows within the pH range of 8.5–11.5 with an optimum at pH 9.5–9.7. Obligately dependent on CO_3^{2-} ion. Grows at a total mineralization of 0.17–3.6 M Na^+ with an optimum of 1–1.87 M Na^+ (in the form of sodium carbonates). Cl^- ion is not required.

Mesophilic. The range of growth temperatures is 18–56°C, the optimum temperature is 38°C. Under optimal growth conditions, the generation time is 3 h.

Facultatively anaerobic. Catalase-positive. During anaerobic growth, ferments glucose, sucrose, maltose, trehalose, cellobiose, melibiose, peptone, yeast extract, and, at a low rate, Tween 80. The products of glucose fermentation are formate, acetate, and ethanol. Starch, glycogen, and xylan are hydrolyzed. Additional substrates utilized during aerobic growth are xylose fructose and lactose. Yeast extract, peptone, and Tween 80 are not utilized aerobically.

Chemoorganotrophic. Exhibits an anabolic requirement for yeast extract (the obligate requirement is methionine). Uses sulfur as an electron acceptor, but sulfur reduction is not coupled to energy generation. High concentrations of sulfide are inhibitory.

The G+C content of DNA is 39.2 mol %.

Isolated from the bottom sediment of a coastal lagoon of Lake Magadi (Kenya).

The type strain is Z-7792^T (=DSM 13870; Uniqem 212).

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